

Biofilm inhibition activity of traditional medicinal plants from Northwestern Argentina against native pathogen and environmental microorganisms

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Abstract

Introduction: Plants have been commonly used in popular medicine of most cultures for the treatment of disease. The in vitro antimicrobial activity of certain Argentine plants used in traditional medicine has been reported. The aim of this study was to investigate the antimicrobial, anti-biofilm, and anti-cell adherence activities of native plants (*Larrea divaricata*, *Tagetes minuta*, *Tessaria absinthioides*, *Lycium chilense*, and *Schinus fasciculatus*) collected in northwestern Argentina. **Methods:** The activities of the five plant species were evaluated in *Bacillus* strains and clinical strains of coagulase-negative *Staphylococcus* isolated from northwestern Argentina and identified by 16S rDNA. **Result:** *Lycium chilense* and *Schinus fasciculatus* were the most effective antimicrobial plant extracts (15.62 µg/ml and 62.50 µg/ml for *Staphylococcus* sp. Mcr1 and *Bacillus* sp. Mcn4, respectively). The highest (66%) anti-biofilm activity against *Bacillus* sp. Mcn4 was observed with *T. absinthioides* and *L. divaricata* extracts. The highest (68%) anti-biofilm activity against *Staphylococcus* sp. Mcr1 was observed with *L. chilense* extract. *T. minuta*, *T. absinthioides*, and *L. divaricata* showed percentages of anti-biofilm activity of between 55% and 62%. The anti-adherence effects of *T. minuta* and *L. chilense* observed in *Bacillus* sp. Mcn4 reflected a difference of only 22% and 10%, respectively, between anti-adherence and biofilm inhibition. Thus, the inhibition of biofilm could be related to cell adherence. In *Staphylococcus* sp. Mcr1, all plant extracts produced low anti-adherence percentages. **Conclusion:** These five species may represent a source of alternative drugs derived from plant extracts, based on ethnobotanical knowledge from northwest Argentina.

Keywords: Anti-biofilm. Traditional medicinal plant. *Bacillus*. *Staphylococcus*.

INTRODUCTION

Resistance to antibiotics has become a major public health problem worldwide as it reduces the effectiveness of treatments and increases morbidity, mortality, and health-care costs⁽¹⁾. This challenge is a natural consequence of the adaption of infectious pathogens to the types of antimicrobials used in multiple areas, including medicines, plants and animals raised as food, and disinfectants used in farms, hospitals, and households⁽²⁾⁽³⁾.

Research on plants as potential sources of new and effective antimicrobials with novel modes of action is well established. Although active constituents may occur in plants in low concentrations, plant extracts may, in many cases, represent a superior source of antimicrobial compounds than synthetic drugs⁽⁴⁾.

The use of plants for the treatment of multiple ailments has been a common practice in the popular medicine of most cultures, even where the precise causation of a disease and/or the mechanisms of its cure have not always been understood⁽⁵⁾⁽⁶⁾. Exploratory research on plants as sources of new antimicrobial compounds has led to a joint interest in fundamental research in the fields of agriculture, chemistry, and medicine. However, studies on the antimicrobial activity of plant extracts have been largely restricted to the analysis of their bacteriostatic and bactericidal properties⁽⁷⁾.

There are some existing reports on the in vitro antimicrobial activity of ethanolic extracts of some Argentine plants used in traditional medicine⁽⁸⁾⁽⁹⁾, although few studies evaluating antimicrobial activity as well as anti-biofilm activity in relation to pathogenic microorganisms have been reported⁽¹⁰⁾⁽¹¹⁾. Biofilm is the predominant mode of growth for bacteria in most natural, industrial, and clinical environments. Biofilms typically consist of densely packed, multi-species populations

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of cells, encased in a self-synthesized polymeric matrix and attached to a tissue or surface⁽¹²⁾. This mode of growth protects bacteria from environmental stresses, that is, the treatment of biofilms with antibiotics or other biocides is often ineffective for eradication⁽¹³⁾. Biofilm formation is therefore a major challenge in many contexts, ranging from industrial corrosion and biofouling⁽¹⁴⁾ to chronic and nosocomial infections⁽¹⁵⁾.

The purpose of the present work was therefore to investigate and demonstrate the presence of antimicrobial, anti-biofilm, and anti-adherence activities in native plants collected in northwestern Argentina (specifically, *Larrea divaricata*, *Tagetes minuta*, *Tessaria absinthioides*, *Lycium chilense*, and *Schinus fasciculatus*), which have been traditionally used for a variety of folk medicinal purposes.

METHODS

Microorganisms and fermentation conditions

Bacillus strains were isolated from a variety of sources in northwestern Argentina, such as soils contaminated with hydrocarbons and natural soils from a riverbank. Soil suspensions were created and heated at 80°C for 15 min, plated onto Luria-Bertani (LB) agar, and incubated at 37°C. Clinical isolates of coagulase-negative *Staphylococcus* (CoNS) were also obtained from a dialysis center in the City of San Miguel de Tucumán. All isolated bacteria were characterized using conventional biochemical methods, namely catalase, bacitracin susceptibility, and coagulase testing. Liquid cultures of *Bacillus* and CoNS were prepared in 125-ml flasks containing 10ml of LB medium on an orbital shaker at 37°C.

Molecular characterization

Total deoxyribonucleic acid (DNA) was extracted from cells harvested in the mid-exponential growth phase as described previously⁽¹⁶⁾. Polymerase chain reaction (PCR) amplification was performed in a 25-μl reaction mix containing 2.5μl 10X STR reaction buffer (Promega, Wisconsin, United States), 20ng total DNA, 0.5μM of each primer, and 1U Taq DNA polymerase (Promega). Primers of the sequence 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to generate partial sequences of 16S ribosomal DNA (rDNA). Amplification conditions were as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C; and 7 min at 72°C for the final extension. PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels.

DNA sequencing was performed by Macrogen Services (Seoul, South Korea). Sequences were compared and aligned with the GenBank database using Basic Local Alignment Search Tool (BLAST) software. Partial nucleotide sequences of the 16S rDNA gene of *Bacillus* sp. and CoNS *Staphylococcus* sp. were deposited in the GenBank database under accession numbers KP872905 and KP872906, respectively.

Plant material and extraction

In the present study, samples of five native plants from four families were collected in order to evaluate their antibacterial

and/or anti-biofilm activities (**Table 1**)^{(17)(18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31)}. The leaves of these plants were collected in the locality of Tafi del Valle in the province of Tucumán in northwestern Argentina (**Figure 1A**). We focused our study on leaves because they represent a sustainable resource and, although bark has been traditionally used, leaf extracts tend to show higher activity. The identity of the plant material was confirmed and voucher specimens were entered into the Miguel Lillo Herbarium at the National University of Tucumán. Botanical and vernacular names, traditional uses, and reported chemical compositions are shown in **Table 1**. The collected leaves were dried at room temperature in a well-ventilated room and ground to fine powder using a mortar and pestle. This powder was then macerated in 80% ethanol for 48h and then filtered through Whatman n° 1 filter paper.

The solid residue was suspended in 50% ethanol until dissolution. The extracts were then concentrated to dryness using a rotary evaporator (Figmay, Córdoba Argentina) under reduced pressure and at a temperature not exceeding 50°C. For antimicrobial and anti-biofilm studies, several concentrations of each prepared powder were evaluated (8-500μg/ml).

Determination of sub-inhibitory concentration and minimum inhibitory concentration

The microorganisms used in this study were cultured in Mueller-Hinton (MH) broth and incubated at 37°C for 24h prior to assays. All cultures were standardized by standard dilution in MH broth (4°C) and characterized by experimental transmittance [$T=30$, optical density (OD)₆₁₀]. Growth at 4°C was stopped by immersion in ice-cold water. From this standard dilution, a 50 ml working suspension was prepared with corrected transmittance $T=95$ (25μl of $T=30$ in 5ml of refrigerated MH broth), corresponding to 1.5×10^8 colony-forming units (CFU)/ml for the *Bacillus* sp. strain and 1.2×10^8 CFU/ml for the *Staphylococcus* sp. strain. This dilution was maintained at 4°C to ensure cellular quiescence. Inoculum (100μl) was added to each well containing sample plant extracts.

The effect of plant extracts on the growth of *Bacillus* and CoNS *Staphylococcus* isolates was evaluated using a standard broth microdilution methodology. Serial dilution was employed to determine the minimum inhibitory concentration (MIC) [the lowest plant metabolite concentration (μg/ml) that inhibited bacterial growth] and sub-inhibitory concentration (SIC) [the highest extract concentration (μg/ml) below the MIC that did not inhibit bacterial growth] concentrations in the range 8-500μg/ml after 24h of growth.

The plant extracts at each concentration were prepared in dimethyl sulfoxide (DMSO) solvent, aliquoted into multi-well polystyrene plates, and dried to allow DMSO evaporation (to avoid the toxic effects of DMSO on bacteria). Negative controls (cells + MH broth), positive controls [cells + MH broth + antibiotics (chloramphenicol and gentamicin)], and media controls (MH) were included. Positive controls for antibiotics were prepared at 8-500μg/ml using serial dilution. All tests were performed in quadruplicate.

Plates were incubated at 37°C for 24h. To determine growth, absorbance was read at 610nm using a microplate reader

TABLE 1

Botanical and common names, traditional uses, and reported chemical compositions of five native plants (*Larrea divaricata*, *Tagetes minuta*, *Tessaria absinthioides*, *Lycium chilense*, and *Schinus fasciculatus*) from Tañi del Valle, Tucumán, northwestern Argentina.

Botanical name	Family	Collection site	Coordinates	Voucher ID	Chemical composition	Ethnobotanical uses
<i>Larrea divaricata</i> Cav.	Zygophyllaceae	Ampimpa (Dpt. Tañi del Valle)	S26° 36,519' W65° 50,678'	Slanis et al. 3427 (LIL)	Lignans, flavonoids as quercetin ⁽¹⁷⁾	Tuberculosis, common cold, antitumor, antifungal ⁽¹⁸⁾
<i>Lycium chilense</i> Miers ex Bertero	Solanaceae	Amaicha (Dpt. Tañi del Valle)	S26° 35,632' W65° 54,953'	Slanis et al. 3429 (LIL)	Alkaloids, nortropanes alkaloids, flavones, flavonoids, glycosylated flavonoids ⁽¹⁹⁾	Antioxidant, antitumor, antibacterial, and antifungal activity ⁽¹⁹⁾
<i>Schinus fasciculatus</i> L.	Anacardiaceae	Amaicha (Dpt. Tañi del Valle)	S26° 35,632' W65° 54,953'	Slanis et al. 3427 (LIL)	Sesquiterpene, terpenoids, flavonoids, saponins, sterols, essential oils, gums and resins ^{(20) (30) (5) (21)}	Antibacterial and antifungal activity ⁽²²⁾
<i>Tagetes minuta</i> L.	Asteraceae	Road 307 km 53 (Dpt. Tañi del Valle)	S26° 53'6,49'' W65° 41'28,40''	Slanis et al. 3125 (LIL)	Essential oils, terpenoids, flavonoids as quercetagenin, patiletin, isorhamnetin ^{(23) (24)}	Antimicrobial ^{(25) (26)} , antioxidant ^{(27) (6)} , repellent ⁽²⁸⁾ , and acaricide activity ⁽²⁹⁾
<i>Tessaria absinthioides</i> (Hook & Arn.) ex DC.	Asteraceae	Los Zazos (Dpt. Tañi del Valle)	S26° 36,189' W65° 54,074''	Slanis et al. 3428 (LIL)	Sesquiterpene, sulfur compounds, flavonoids, essential oils ⁽³⁰⁾	Antiviral activity ⁽³¹⁾

(Thermo Scientific™ Multiskan™ GO Ultraviolet-Visible microplate spectrophotometer, United States). To determine the effects of the extracts, a formula was used to calculate percent inhibition. The mean percentage of inhibition from the replicates was used to determine the final MIC and SIC values⁽⁷⁾.

$$\% \text{ Inhibition} = \left(1 - \left(\frac{(\text{OD}_{24} - \text{OD}_{10})}{\text{OD}_{\text{gc}_{24}} - \text{OD}_{\text{gc}_{10}}} \right) \right) \times 100$$

OD₂₄ = optical density (610 nm) of the test well at 24 hours post-inoculation

OD₁₀ = optical density (610 nm) of the test well at 0 hours post-inoculation

OD_{gc₂₄} = optical density (610 nm) of the growth control well at 24 hours post-inoculation OD_{gc₁₀} = optical density (610 nm) of the growth control well at 0 hours post-inoculation

Biofilm formation and anti-biofilm effects of plant extracts

Biofilm production was carried out with the method in polystyrene multi-well cell culture plates. The biofilm-producing CoNS *Staphylococcus* strains 263 and 1174 (isolated from bovine mastitis and provided by the Faculty of Veterinary Medicine, Buenos Aires University) and a non-producing CoNS *Staphylococcus* strain (ATCC 12228) were used as controls⁽¹⁾.

The inoculum of each microorganism was standardized through standard dilution with LB (4°C) and characterized by experimental transmittance (T=30, OD₆₁₀). From this standard dilution, a 50ml working suspension was prepared with corrected transmittance T=95 (25µl of T=30 in 5ml of LB broth at 4°C) corresponding to 9 × 10⁸ CFU/ml for *Bacillus* sp. strain and 1.6 × 10⁸ CFU/ml for *Staphylococcus* sp. strain. To detect biofilm-producing bacteria, 100µl of inoculum from each bacterial suspension and medium control (LB broth) were deposited in triplicate on multi-well polystyrene plates, and the plates were incubated at 37°C for 24h. After incubation,

medium was extracted by inversion and wells were washed twice with phosphate-buffered saline (PBS) (8.1mM Na₂HPO₄, 1.5mM KH₂PO₄, 140mM NaCl, and 3mM KCl; pH 7.2). The contents of wells were emptied by inversion and plates were dried upside-down on absorbent paper. Once dry, biofilms appeared as whitish, opaque membranes and were fixed with methanol for 7 min⁽³²⁾. Plates were emptied again by inversion and air-dried. Once dry, an aqueous solution of 0.1% safranin was used as a stain. After 4 min, plates were again inverted, washed with water, dried upside-down⁽¹⁾.

Biofilm formation was evaluated using a microplate reader at 490nm (Thermo Scientific™ Multiskan™ GO Ultraviolet-Visible microplate spectrophotometer, United States). The strains considered as the best producers of biofilm were those with OD₄₉₀ values greater than those of the control strains (OD₄₉₀ > 0.08)⁽¹⁾.

Next, the selected bacterial strains were analyzed in the absence and presence of plant extracts. To determine the effects of plant extracts on biofilm formation, SIC of plant extracts (10-100µg/ml as appropriate for each microorganism) were added to the test wells and allowed to dry prior to inoculation. Inoculum (corresponding to 9×10^8 CFU/ml for *Bacillus* sp. strain and 1.6×10^8 CFU/ml for *Staphylococcus* sp. strain) was added in the form of a bacterial suspension at the zero hour time point of biofilm formation, and biofilms were allowed to develop for 24h as described above. Each bacterial strain inoculated in the absence of a plant extract was considered as a positive control for biofilm formation.

Bacterial adhesion assay

Inoculum was prepared as a 1:10 dilution of microorganism cultures (24h) using fresh LB broth followed by incubation at 37°C for 2h with shaking at 100rpm (inoculum had OD₆₁₀=0.100, corresponding to 1.5×10^8 CFU/ml). Inoculum of 200µl was then added to multi-well polystyrene plates at 37°C for 3h in the presence and absence of plant extracts at SIC. Next, the medium was extracted by inversion, plates were washed twice with PBS, and bacterial adhesions were fixed with 25% formaldehyde for 30 min. Wells were then dried and stained with 0.1% safranin. Cell adherence measurement was carried out in a microplate reader at 490nm. The percentage of inhibition of bacterial adhesion was determined as described above.

Statistical analysis

Statistical analysis was performed using SigmaPlot 12.0 and Minitab (Minitab, Inc.) software version 14 for Windows. Normality distribution was evaluated by Shapiro-Wilk test and analysis of variance (ANOVA) was used to evaluate the mean differences among treatments. Two-way ANOVA and subsequent comparisons were performed using the Holm-Sidak test and Bonferroni post-test as appropriate. Results were presented as the mean Standard Deviation (SD), and differences were accepted as significant for $p < 0.05$ (5% of the significance level).

RESULTS

Isolation and selection of indigenous spore-forming bacteria and coagulase-negative *Staphylococcus* strains

A total of 53 spore-forming bacteria were isolated from natural environments, and 22 CoNS strains were isolated from

a dialysis center⁽¹⁾. From this total of 75 native isolates, 24 were selected as biofilm producers and the Mcn4 and Mcr1 strains were identified as the best biofilm producers compared with control strains (**Figure 1B**). Partial sequencing of the 16S rDNA gene confirmed the biochemical and morphological characterization of both strains. A BLAST search and alignment analysis showed a similarity of 99% for strain Mcn4 with *Bacillus licheniformis*, hereinafter referred to as *Bacillus* sp. Mcn4; and strain Mcr1 with *Staphylococcus sciuri*, hereinafter referred to as *Staphylococcus* sp. Mcr1.

Determination of sub-inhibitory concentration and minimum inhibitory concentration

Table 2 shows the SIC and MIC values obtained for the five plant extracts. These results reflect a variable degree of antibacterial activity against the *Bacillus* and *Staphylococcus* genera, and indicate that dose-dependent antimicrobial activity was observed for all plant extracts.

Extracts from *L. chilense* and *S. fasciculatus* showed the best antimicrobial properties, with SIC values of 62.50µg/ml for *Bacillus* sp. Mcn4 and 15.62µg/ml for *Staphylococcus* sp. Mcr1 for both extracts (**Table 2**). Furthermore, both *L. chilense* and *S. fasciculatus* showed antimicrobial activity against *Bacillus* strains, with an SIC value of 62.50µg/ml, the same SIC observed for chloramphenicol (**Table 2**). The antimicrobial activities shown by the genera *Schinus* and *Lycium* against *Staphylococcus* sp. Mcr1 included SIC values equal to those observed for gentamicin (15.62µg/ml).

A moderate level of antimicrobial activity (SIC 125-250µg/ml) was observed against *Bacillus* sp. Mcn4 for *T. minuta*, *T. absinthioides*, and *L. divaricata* (**Table 2**). However, these plant extracts showed high antimicrobial activity against *Staphylococcus* sp. Mcr1. It is notable that all plant extracts

TABLE 2

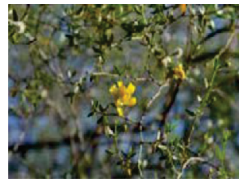
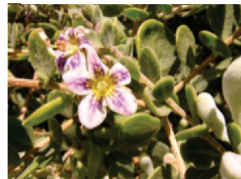
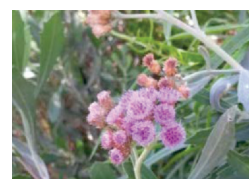
Effects of *Larrea divaricata*, *Tagetes minuta*, *Tessaria absinthioides*, *Lycium chilense*, and *Schinus fasciculatus* on the viability of bacteria from the genera *Bacillus* and *Staphylococcus*.

Plant name	<i>Bacillus licheniformis</i>		<i>Staphylococcus sciuri</i>	
	MIC ¹ (µg/ml)	SIC ²	MIC ¹ (µg/ml)	SIC ²
<i>Schinus fasciculatus</i>	125	62.50	31.25	15.62
<i>Tagetes minuta</i>	500	250	62.5	31.25
<i>Tessaria absinthioides</i>	250	125	125	62.50
<i>Lycium chilense</i>	125	62.50	31.25	15.62
<i>Larrea divaricata</i>	250	1250	31.25	15.62
<i>Gentamicin</i>		15.62		15.62
<i>Chloramphenicol</i>		62.50		62.50

MIC (µg/ml) and SIC (µg/ml) were determined, with values expressed as means from triplicate assays. ¹MIC = the lowest plant metabolite concentration (µg/ml) that inhibited bacterial growth. ²SIC = the highest extract concentration (µg/ml) below the MIC that did not inhibit bacterial growth. **MIC**: minimum inhibitory concentration; **SIC**: sub-inhibitory concentration.

(A)

Natives plants in northwestern Argentina

*Tagetes minuta**Larrea divaricata**Schinus fasciculatus**Lycium chilense**Tessaria absinthioides*Antimicrobial activity
(MIC and SIC)

Anti-adherence activity

Anti-biofilm activity

(B)

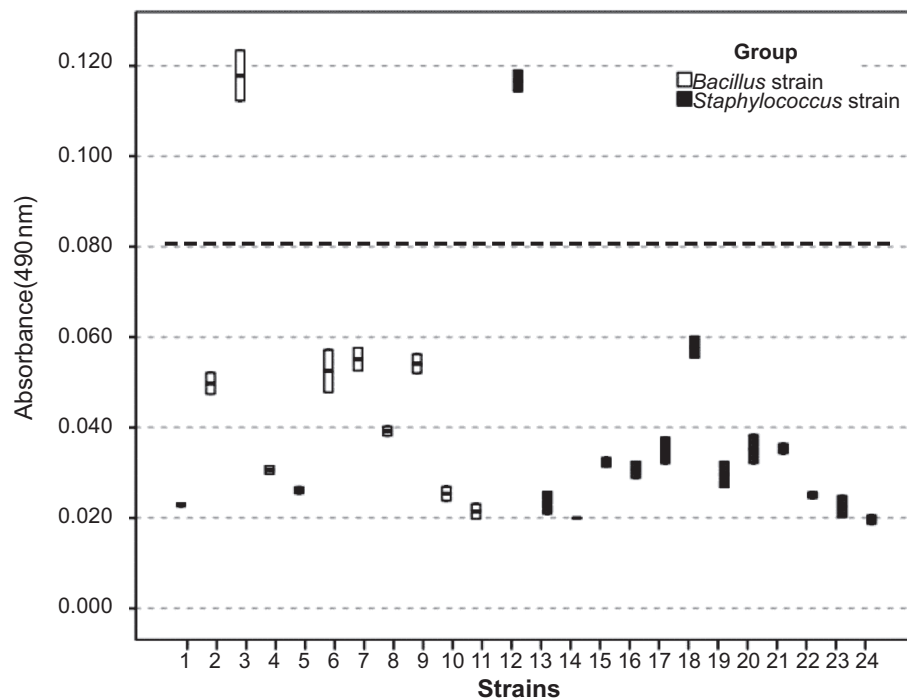
Biofilm producers from the genera *Bacillus* and *Staphylococcus*

FIGURE 1. (A): Anti-microbial and anti-biofilm activity of medicinal plants from Northwestern Argentina against native isolates from the genera *Bacillus* and *Staphylococcus*. **(B):** A total of 53 spore-forming bacteria were isolated from natural environments and 22 coagulase-negative *Staphylococcus* (CoNS) were isolated from a dialysis center. Of these, 24 were selected as biofilm producers. The best biofilm producing strains were identified as *Bacillus* sp.+ Mcn4 (strain 3) and *Staphylococcus* sp. Mcr1 (strain 12) ($p < 0.01$). **MIC:** minimum inhibitory concentration; **SIC:** sub-inhibitory concentration.

showed SIC values between 15.62-62.5µg/ml against this microorganism.

The *L. divaricata* extract showed remarkable activity against the genus *Staphylococcus*, with an SIC value equal to that of gentamicin (15.62µg/ml), which was used as the reference antibiotic. Furthermore, *T. absinthioides* extract showed similar antimicrobial activity against *Staphylococcus* sp. Mcr1 to that of chloramphenicol (Table 2).

Effect of plant extracts on biofilm formation

The anti-biofilm activity of the plant extracts on *Bacillus* sp. Mcn4 and *Staphylococcus* sp. Mcr1 was also tested at selected SIC values.

The best anti-biofilm activity (66%) against *Bacillus* sp. Mcn4 was observed with the extract from *T. absinthioides* at 100µg/ml (Figure 2A) and with *L. divaricata* extract across the entire range evaluated (10-100µg/ml), with no significant differences observed for the latter extract across the extract concentrations evaluated ($p = 0.67$) (Figure 2A).

Lycium chilense showed biofilm inhibition of 45% and 47% at 10µg/ml and 50µg/ml, respectively, and the difference between these two concentrations was not significant ($p = 0.09$). With *T. minuta* extract, the greatest inhibition (50%) was observed at 100µg/ml, while for *S. fasciculatus* extract, no significant biofilm inhibition was observed (Figure 2A).

Anti-biofilm activity against *Staphylococcus* sp. Mcr1 was also evaluated at selected SIC values (Figure 2B). The highest anti-biofilm activity was observed with *L. chilense* extract (68%). In this case, anti-biofilm activity was dose dependent, with the highest inhibition observed at 50µg/ml ($p = 0.02$). *Tagetes minuta*, *T. absinthioides*, and *L. divaricata* showed percentages of anti-biofilm activity with values between 55% and 62% (Figure 2B). For these extracts, no significant difference was observed between the two concentrations evaluated (10µg/ml and 50µg/ml: $p = 0.17$, $p = 0.05$, and $p = 0.59$ for *T. minuta*, *T. absinthioides*, and *L. divaricata*, respectively). As also observed against the genus *Bacillus*, *S. fasciculatus* extract showed only a low level of biofilm inhibition activity (Figure 2B).

Effect of plant extracts on cell adherence

The anti-adherence effects of the plant extracts were also evaluated. Samples selected for testing included the plant extract concentrations that had previously shown the most effective inhibition of biofilm (Figure 2).

The anti-adherence effects of *T. absinthioides* and *L. divaricata* extracts against *Bacillus* sp. Mcn4 were found to be lower than their anti-biofilm activity, indicating that their ability to inhibit biofilm formation took place during a step subsequent to cell adherence (Figure 3A). The percentage of anti-adherence observed with *T. minuta* and *L. chilense* extracts reflected a difference only of 22% and 10%, respectively, between anti-adherence and biofilm inhibition (Figure 3A).

Schinus fasciculatus showed the same percentage of anti-adherence effect as its percentage of anti-biofilm activity, with no significant difference observed between these ($p = 0.55$). Thus, for this plant extract, the inhibition of biofilm formation could be related to cell adherence.

In contrast, all plant extracts tested showed low anti-adherence percentages against *Staphylococcus* sp. Mcr1, suggesting that their biofilm inhibition abilities may be related to a later step in biofilm formation. In all of these cases, the anti-biofilm effect was more than 50% higher than the anti-adherence effect (Figure 3B).

DISCUSSION

The more potent antimicrobial activities observed for plant extracts were against the *Staphylococcus* strain. The antimicrobial activities produced by extracts of the genera *Schinus* and *Lycium* against *Staphylococcus* sp. Mcr1 were comparable to those observed for gentamicin. This antimicrobial effect of the genus *Schinus* against *Staphylococcus* was also observed by Yao et al.⁽¹⁹⁾ and Gehrke et al.⁽³³⁾, with both of these sources reporting an MIC value of 125µg/ml. However, in the present work, a more effective MIC of 31.25µg/ml (Table 2) was observed. The similarities in the activities observed for *S. fasciculatus* and *L. chilense* could be due to the presence of flavonoids in both extracts. The antibacterial properties of these compounds are increasingly being reported, and raw extracts from plants with a history of use in traditional medicine have now been screened for antimicrobial activities by numerous research groups⁽³⁴⁾⁽³⁵⁾. However, it is also possible that the high antimicrobial activity shown by *S. fasciculatus* was due to disruption of the bacterial membrane caused by the presence of terpenoids, which inhibit Adenosine triphosphate (ATP) synthesis⁽³⁶⁾⁽³⁷⁾.

However, ethanolic extract of *L. divaricata* showed potent antimicrobial activity against *Staphylococcus* sp. Mcr1. Davicino et al.⁽¹⁸⁾ have reported that the fraction obtained from an ethanolic extract of *L. divaricata* contained several phenolic compounds, which may be responsible for the cytotoxicity observed against the *Staphylococcus* genus.

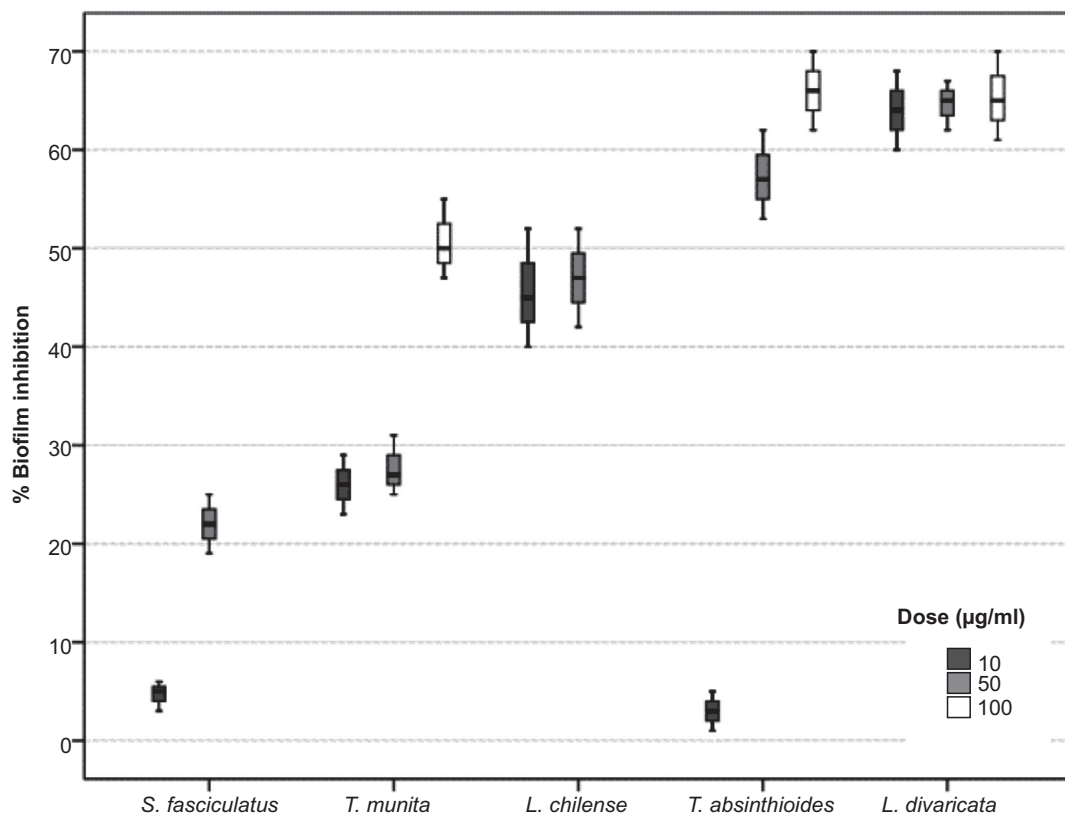
The antimicrobial activity observed with *T. minuta* and *T. absinthioides* may be due to the presence, not only of flavonoids, but also of essential oils of the family *Asteraceae* (to which these species belong; Table 1), which have been reported as compounds with antimicrobial activity⁽³⁸⁾⁽³⁹⁾.

The SIC values determined for each plant extract were used to evaluate biofilm inhibition.

Biofilm eradication is difficult to achieve because of host defenses and inherent resistance to antibiotics and biocides. Furthermore, there are several mechanisms used to explain the resistance of biofilms to antimicrobials⁽⁴⁰⁾, which makes it difficult to predict the behavior of biofilm cells. In the present study, the anti-biofilm activity of plant extracts at SIC was evaluated after 24h and the percentage of biofilm inhibition were observed (Figure 2). By comparing the values obtained, it can be seen that plant extract concentrations significantly affected the removal and/or inactivation of biofilms.

Extracts from these medicinal plants may influence biofilm formation by damaging microbial membrane structures⁽⁴⁾, inhibiting peptidoglycan synthesis, and/or modulating quorum sensing⁽⁴¹⁾. With regard to the last of these mechanisms, several approaches involving quorum interference have

(A)



(B)

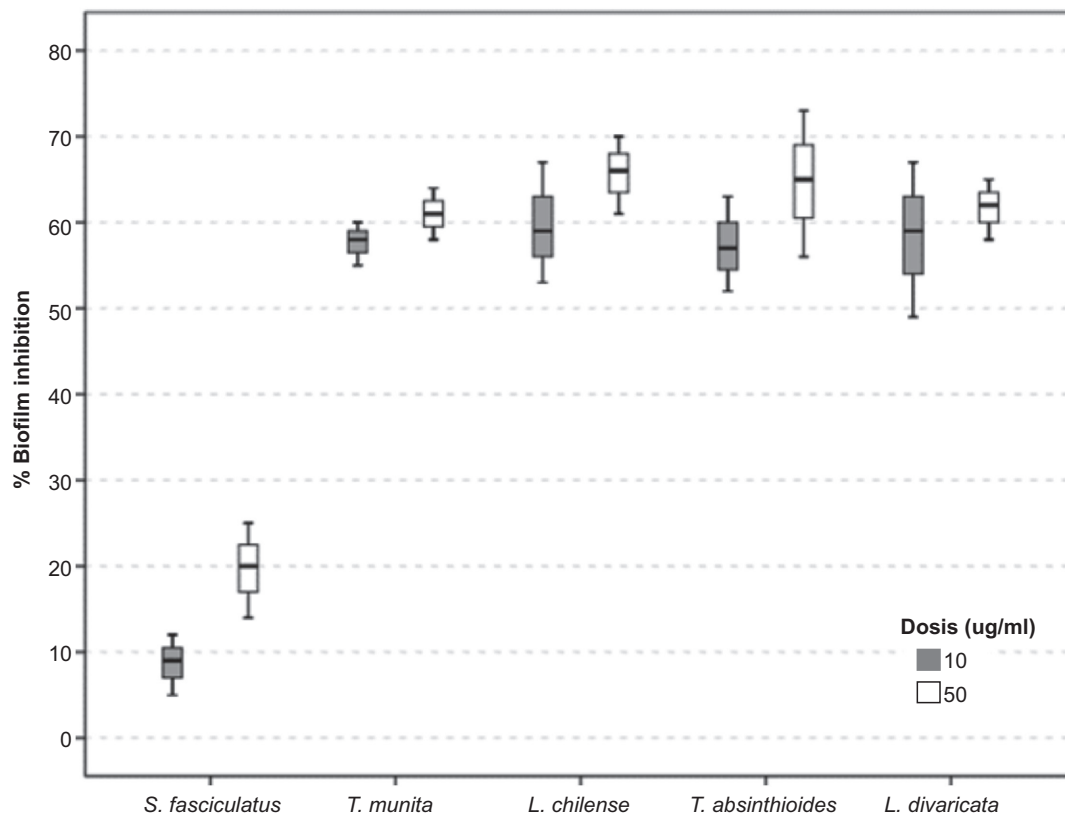


FIGURE 2. Anti-biofilm effects of extracts of traditional medicinal plants from Northwestern Argentina against. (A): *Bacillus* sp. Mcn4 and (B): *Staphylococcus* sp. Mcr1. Sub-inhibitory concentrations from 10-100µg/ml were used. The values are expressed as percentages of bacterial biofilm inhibition by each plant extract, compared with the control (no plant extract) and expressed as means \pm standard deviation of triplicate assays.

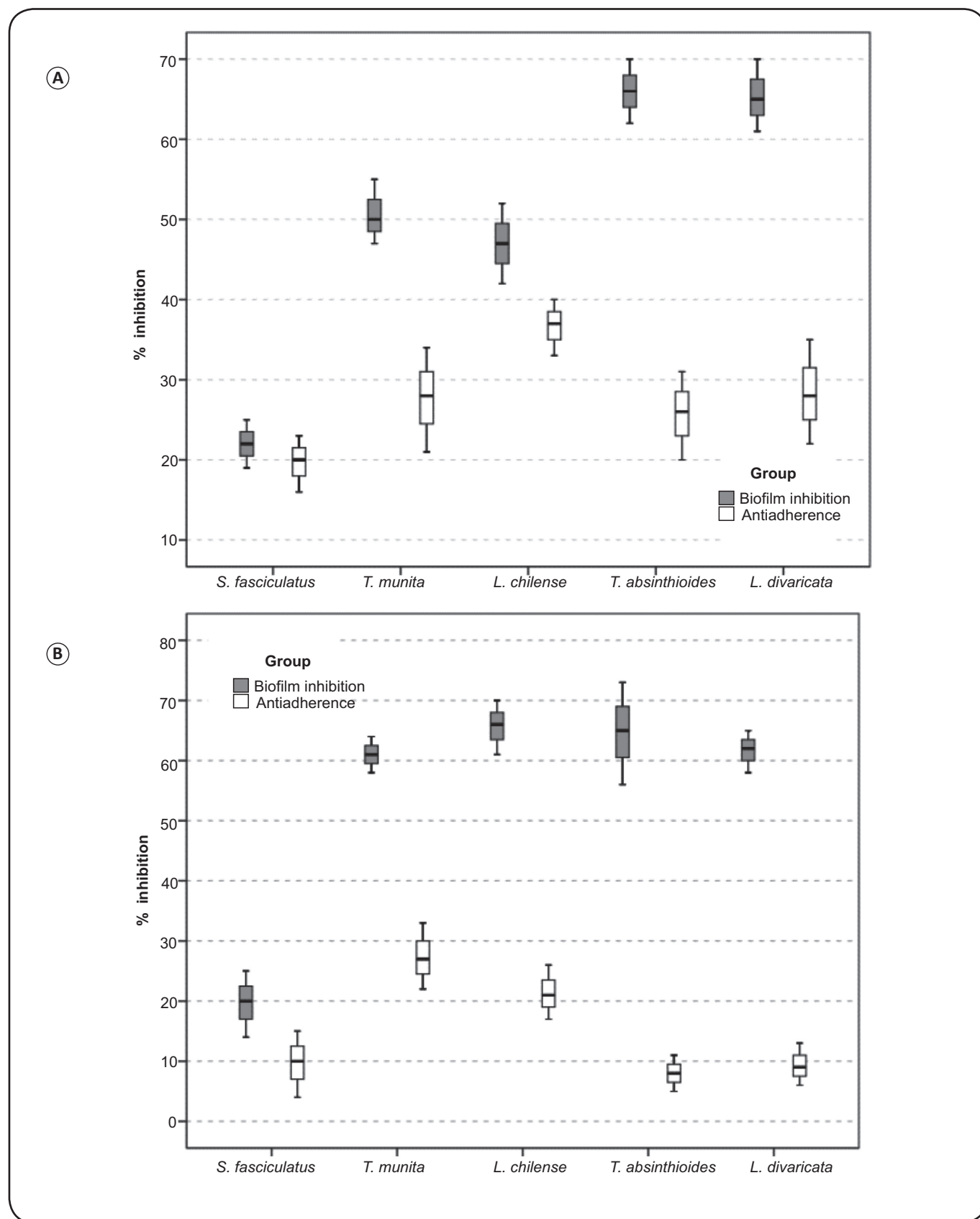


FIGURE 3. Anti-adherence effects against (A): *Bacillus* sp. Mcn4 and (B): *Staphylococcus* sp. Mer1 cells. Values are expressed as percentages of cell anti-adherence inhibition compared with anti-biofilm inhibition. The values are expressed as means \pm standard deviation of triplicate assays.

been studied and they represent the most recent strategies to counteract staphylococcal infections, among others. Quorum interference can be achieved by several alternative strategies, including quorum sensing disruption through inhibition of signal molecule biosynthesis, signal molecule inactivation, and blockage of signal transduction^{(41) (42)}. In fact, flavonoids are among the compounds that exert anti-biofilm effects via quorum sensing inhibition⁽⁴³⁾. In the present study, all plant extracts that showed biofilm inhibition contain flavonoids, and as such, these compounds may be responsible for the biofilm inhibition observed, although further studies are required to investigate this.

Biofilm formation occurs stepwise, and one step of relevant importance is cell adhesion that occurs prior to biofilm formation⁽⁴⁴⁾. In view of the importance of this step in biofilm production, the anti-adherence effects of the plant extracts under study were also evaluated.

For *S. fasciculatus*, the inhibition of biofilm formation observed may be related to cell adherence. Considering that adherence is a prerequisite for colony formation, preventing bacterial adhesion would appear to be an ideal strategy for reducing biofilm formation⁽⁴⁴⁾.

For the plant extracts evaluated in this study, anti-biofilm and anti-adherence properties against the genus *Bacillus* and anti-biofilm activity against CoNS isolates have not been previously reported. These findings may represent novel, alternative approaches to biofilm control, especially in relation to infectious diseases caused by CoNS⁽¹⁾. Furthermore, the biodeterioration of metals in industrial processes has detrimental effects on the environment with economic implications. The production of biofilm by *Bacillus* spp. affects the level of metal corrosion in industrial processes⁽⁴⁵⁾. Thus, the development of a preventive strategy against bacterial-mediated corrosion is necessary.

The plant extracts studied here showed antimicrobial activity as well as biofilm inhibition against the genera *Bacillus* and *Staphylococcus*. These findings may represent the first of many steps towards the development of new antimicrobial and anti-biofilm drugs using extracts from plants used in traditional folk medicine in northwestern Argentina. Given the importance of developing new strategies for controlling the growth of biofilm because of its pathogenic association with several diseases or influence on the level of metal corrosion in industrial processes, further investigation of the natural biofilm inhibition exhibited by Argentinean plant extracts is clearly warranted.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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REFERENCES

- de Allori MCG, Jure MA, Romero C, de Castillo MEC. Antimicrobial resistance and production of biofilms in clinical isolates of coagulase-negative *Staphylococcus* strains. *Biol Pharm Bull* 2006; 29:1592-1596.
- Bloomfield SF. Significance of biocide usage and antimicrobial resistance in domiciliary environments. *J Appl Microbiol* 2002; 92:144-157.
- Monte J, Abreu AC, Borges A, Simões LC, Simões M. Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens* 2014; 3:473-498.
- Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmingto JR, et al. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol* 2000; 88:170-175.
- Erazo S, Delporte C, Negrete R, Garcia R, Zaldivar M, Ittura G, et al. Constituents and biological activities of *Schinus molle*. *J Ethnopharmacol* 2006 107:395-400.
- Pérez RM, Hernández H, Hernández S. Antioxidant activity of *Tagetes erecta* essential oil. *J Chil Chem Soc* 2006; 51:883-886.
- Quave CL, Plano LRW, Pantuso T, Bennett BC. Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*. *J Ethnopharmacol* 2008; 118:418-428.
- Tereschuk ML, Baigori MD, de Figueroa LIC, Abdala LR. Flavonoids from Argentine species of *Tagetes* (Asteraceae) with antimicrobial activity. *Methods Mol Biol* 2004; 268:317-330.
- Zampini IC, Cudmani N, Isla MI. Actividad antimicrobiana de plantas medicinales argentinas sobre bacterias antibiótico-resistentes. *Acta Bioq Clin Latinoam* 2007; 41:385-393.
- Vacheva A, Mustafa B, Staneva J, Marhova M, Kostadinova S, Todorova M, et al. Effects of extracts from medicinal plants on biofilm formation by *Escherichia coli* urinary tract isolates. *Biotechnol Biochem* 2011; 25 (suppl):92-97.
- Hobby GH, Quave CL, Nelson K., Compadre CM, Beenken KE, Smeltzer MS. *Quercus coccinea* extracts limit *Staphylococcus aureus* biofilm formation. *J Ethnopharmacol* 2012; 144:812-815.
- Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 1987; 41:435-464.
- Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. *Cell Microbiol* 2009; 11:1034-1043.
- Lopez D, Vlamakis H, Kolter R. Biofilms. *Cold Spring Harb Perspect Biol* 2010; 2:a000398.
- Rendueles O, Kaplan JB, Ghigo JM. Antibiofilm polysaccharides. *Environ Microbiol* 2013; 15:334-346.
- Miller JH. Experiments in molecular genetics. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor; 1972. 466p.
- Palacio L, Lloret CV, Baeza C, Ortiz L, Brunetti P, Cantero JJ, et al. Determinación de metabolitos de interés farmacológico en plantas micropropagadas de *Larrea divaricata* Cav. *Bol Latinoam y del Caribe Plant Med y Aromat* 2007; 6:405-406.

18. Davicino R, Martino R, Anesini C. *Larrea divaricata* Cav: scientific evidence showing its beneficial effects and its wide potential application. Bol Latinoam y del Caribe Plantas Med y Aromat 2011; 10:92-103.
19. Yao X, Peng Y, Xu LJ, Li L, Wu QL, Xiao PG. Phytochemical and biological studies of *Lycium* medicinal plants. Chem Biodivers 2011; 8:976-1010.
20. Yuequin Z, Recio MC, Manez S, Giner RM, Cerda-Nicolas M, Rios JL. Isolation of two triterpenoids and a biflavone with anti-inflammatory activity from *Schinus molle* fruit. Planta Med 2003; 69:893-898.
21. Díaz C, Quesada S, Brenes O, Aguilar G, Ciccio JF. Chemical composition of *Schinus molle* essential oil and its cytotoxic activity on tumour cell lines. Nat Prod Res 2008; 22:1521-1534.
22. Gupta MP. 270 Plantas medicinales iberoamericanas. Talleres de Editorial Presencia Ltda; Santa Fe de Bogotá: Colombia; 1995. p. 347.
23. Tereschuk ML, Riera MVQ, Castro GR, Abdala LR. Antimicrobial activity of flavonoids from leaves of *Tagetes minuta*. J Ethnopharmacol. 1997; 56:227-232.
24. Scrivanti RL, Zunino MP, Zygadlo JA. *Tagetes minuta* and *Schinus areira* essential oils as allelopathic agents. Biochem Syst Ecol 2003; 31:563-572.
25. Srinivasan D, Nathan S, Suresh T, Permalsamy PL. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. J Ethnopharmacol. 2001; 74:217-220.
26. Lima B, Aguero MB, Zygadlo J, Tapia A, Solis C, Rojas De Arias A, et al. Antimicrobial activity of extracts, essential oil and metabolites obtained from *Tagetes mendocina*. J Chil Chem Soc 2009; 54:68-72.
27. Schmeda-Hirschmann G, Tapia A, Theoduloz C, Rodríguez J, Lopez S, Feresin GE. Free radical scavengers and antioxidants from *Tagetes mendocina*. Z Naturforsch C 2004; 59:345-353.
28. Gleiser RM, Bonino MA, Zygadlo JA. Repellence of essential oils of aromatic plants growing in Argentina against *Aedes aegypti* (Diptera: Culicidae). Parasitol Res 2011; 108:69-78.
29. García MV, Matias J, Cavalcante Barros J, Pires de Lima D, da Silva Lopes R, Andreotti R. Chemical identification of *Tagetes minuta* Linnaeus (Asteraceae) essential oil and its acaricidal effect on ticks. Rev Bras Parasitol Vet 2012; 21:405-411.
30. García CC, Talarico L, Almeida N, Colombres S, Duschatzky C, Damonte EB. Virucidal activity of essential oils from aromatic plants of San Luis, Argentina. Phytother Res 2003; 17:1073-1075.
31. Visintini Jaime MF, Redko F, Muschietti LV, Campos RH, Martino VS, Cavallaro LV. *In vitro* antiviral activity of plant extracts from *Asteraceae* medicinal plants. Virology J 2013; 10:245.
32. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985; 27: 956-1006.
33. Gehrke ITS, T Neto A, Pedroso M, Mostardeiro CP, Da Cruz IBM, Silva UF, et al. Antimicrobial activity of *Schinus lentiscifolius* (Anacardiaceae). J Ethnopharmacol 2013; 148:486-491.
34. Aristimuño Ficooseco ME, Vattuone MA, Audenaert K, Catalán CA, Sampietro DA. Antifungal and antimycotoxigenic metabolites in Anacardiaceae species from northwest Argentina: isolation, identification and potential for control of *Fusarium* species. J Appl Microbiol. 2014; 116:1262 -1273.
35. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005; 26:343-356.
36. Gyawali R, Ibrahim SA. Impact of plant derivatives on the growth of foodborne pathogens and the functionality of probiotics. Appl Microbiol Biotechnol 2012; 95:29-45.
37. Morán A, Gutiérrez S, Martínez-Blanco H, Ferrero MA, Monteagudo-Mera A, Rodríguez-Aparicio LB. Non-toxic plant metabolites regulate *Staphylococcus* viability and biofilm formation: a natural therapeutic strategy useful in the treatment and prevention of skin infections. Biofouling 2014; 30:1175-1182.
38. Ali NA, Sharopov FS, Al-Kaf AG, Hill GM, Arnold N, Al-Sokari SS, et al. Composition of essential oil from *Tagetes minuta* and its cytotoxic, antioxidant and antimicrobial activities. Nat Prod Commun 2014; 9:265-268.
39. Kouidhi B, Mohammed Y, Qurashi AA, Chaieb K. Drug resistance of bacterial dental biofilm and the potential use of natural compounds as alternative for prevention and treatment. Microb Pathog 2015; 80:39-49.
40. Sillankorv S, Oliveira R, Vieira MJ, Sutherland I, Azeredo J. Bacteriophage Φ S1 infection of *Pseudomonas fluorescens* planktonic cells versus biofilms. J Biofouling 2004; 20:133-138.
41. Hartmann A, Rothballer M, Hense BA, Schröder P. Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. Front Plant Sci 2014; 5:131.
42. Rampioni G, Leoni L, Williams P. The art of antibacterial warfare: deception through interference with quorum sensing-mediated communication. Bioorg Chem. 2014; 55:60-68.
43. Manner S, Skogman M, Goeres D, Vuorela P, Fallarero A. Systematic exploration of natural and synthetic flavonoids for the inhibition of *Staphylococcus aureus* biofilms. Int J Mol Sci 2013; 14:19434-19451.
44. Kostakioti M, Hadjifrangiskou M, Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Harb Perspect Med 2013; 3:a010306.
45. Lin J, Madida BB. Biofilms affecting progression of mild steel corrosion by Gram positive *Bacillus* sp. J Basic Microbiol 2015; 55:1168-1178.